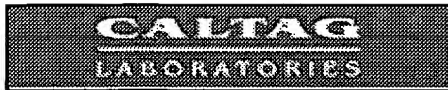


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Topical FK506 Suppresses Cytokine and Costimulatory Molecule Expression in Epidermal and Local Draining Lymph Node Cells During Primary Skin Immune Responses

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Abstract

Recently, it has been shown that the immunosuppressive macrolide lactone, FK506, exerts good therapeutic efficacy in inflammatory skin diseases. The aim of this study was to analyze the influence of topical FK506 on molecular (IL-1 α , IL-1 β , IL-2, IL-4, IL-12 p35, IL-12 p40, macrophage inflammatory protein-2 (MIP-2), granulocyte-macrophage CSF (GM-CSF), TNF- α , and IFN- γ) and cellular (I-A⁺/CD80⁺, I-A⁺/CD54⁺, I-A⁺/CD69⁺, I-A⁺/B220⁺, and CD4⁺/CD25⁺) events in epidermal (EC) and local draining lymph node (LNC) cells during primary contact hypersensitivity responses. Cytokine mRNA levels for IL-1 α , IL-1 β , GM-CSF, TNF- α , MIP-2, and IFN- γ in EC and for IL-2, IL-4, IL-12 p35, IL-12 p40, and IFN- γ in LNC were increased and resulted in significant LNC proliferation during oxazolone-induced contact hypersensitivity. Topical FK506 treatment dose-dependently suppressed oxazolone-induced LNC proliferation. This effect was correlated with decreased IL-1 α , IL-1 β , GM-CSF, TNF- α , MIP-2, and IFN- γ mRNA expression within the epidermis and decreased IL-12 p35 and p40 mRNA expression in LNC. Further analysis of the LNC cytokine pattern revealed that the production of both Th1 (IFN- γ and IL-2) and Th2 (IL-4) cytokines was dramatically impaired after topical FK506 treatment. Flow cytometric analysis showed that topical FK506 decreased

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the population of epidermis-infiltrating CD4⁺ T cells and suppressed the expression of CD54 and CD80 on I-A⁺ EC and LNC during hapten-induced contact hypersensitivity. Furthermore, topical FK506 profoundly impaired oxazolone-induced up-regulation of CD25 expression on CD4⁺ LNC and dramatically decreased hapten-induced expansion of I-A⁺/B220⁺ and I-A⁺/CD69⁺ LNC subsets. In conclusion, these results give new insights into the mechanisms of action of topical FK506 treatment.

► Introduction

FK506 (tacrolimus), a hydrophobic macrolide lactone produced by *Streptomyces tsukubaensis*, exerts profound immunosuppressive effects in vitro and in vivo (1, 2, 3). In dermatopharmacology there is a large demand for alternative nonsteroidal anti-inflammatory agents. Thus, immunomodulatory drugs, preferentially macrolide lactones, have attracted special interest. Cyclosporin A has, in fact, revolutionized the treatment of various severe inflammatory skin diseases but, unfortunately, it lacks activity upon topical application (4). In contrast, topical FK506 exerts good therapeutic efficacy in atopic eczema (5, 6, 7). During recent years, we have been interested in the action of FK506 in the treatment of inflammatory skin diseases, such as psoriasis, atopic eczema, and contact allergy (8, 9, 10). In psoriatic keratinocytes, IL-8 and IL-8R are dramatically overexpressed. Treatment with FK506 down-regulates IL-8 and IL-8R expression in keratinocytes both in vitro and in vivo (11, 12). Furthermore, studies have been focused on the immunosuppressive effects of topical FK506 in experimental human and animal contact hypersensitivity. Moreover, we previously demonstrated percutaneous absorption and dose-dependent suppression of contact hypersensitivity reactions in humans by topical FK506 treatment (10, 13, 14, 15).

Contact hypersensitivity has served as a useful model for primary activation of T cells in skin and skin-associated lymphoid tissue, such as local draining lymph nodes. During contact hypersensitivity, APC of the skin take up Ag, mature, and migrate to local draining lymph nodes where they present relevant Ag determinants together with costimulatory molecules to T cells and induce Ag-specific T cell activation (reviewed in 16).

The aim of this study was to analyze the immunosuppressive mechanisms of action of topical FK506 in vivo. For this purpose, we analyzed the capacity of topical FK506 to interfere with 1) cytokine networks, 2) maturation of APC, 3) T cell activation, 4) T cell migration, and 5) associated B cell activation in skin and local draining lymph nodes during primary contact hypersensitivity responses. Hence, we evaluated both molecular and cellular events during the induction phase of contact hypersensitivity, with special emphasis on the interaction between skin and skin-associated lymphoid tissue.

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Animals

Female NMRI mice were purchased from Winkelmann (Borken, Germany) and accustomed to the new UV-free environment for at least 7 days. All animals were maintained on a standard diet and water ad libitum; they were 6 to 12 wk old at the onset of the experiments.

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Contact hypersensitivity model and immunosuppression

Previous studies showed that optimal sensitization and hapten-induced activation of skin-draining lymph node cells were obtained after sensitizer treatment on 3 consecutive days (17, 18, 19, 20). Therefore, five female NMRI mice per group were topically treated on the dorsal surfaces of both ears with 25 μ l of 0.01 to 1% FK506 (Fujisawa Pharmaceutical, Osaka, Japan) or vehicle (a mixture of 40% dimethylacetamide, 30% acetone, and 30% ethanol) alone on 4 consecutive days. During the last 3 days, mice received an additional topical treatment with the model contact sensitizer oxazolone (1%; 4-ethoxymethylen-2-phenyloxazol-5-one; Sigma, St. Louis, MO). On day 4, mice were sacrificed, and ears and auricular lymph nodes from each animal were removed and pooled. Epidermal cell (EC)² suspensions were harvested after trypsin treatment of ear skin, and lymph node cell (LNC) suspensions were prepared by mechanical tissue disaggregation through a sterile stainless gauze. Single cell suspensions were used to determine LNC proliferation, to perform flow cytometric analysis of cell surface markers, and to extract total RNA. Cell viability was checked by trypan blue staining and ranged between 90 and 95%. To analyze the effects of topical FK506 early during the induction of contact hypersensitivity, 12 h after the first contact sensitizer treatment mice were sacrificed, and ears and local draining lymph nodes were removed and analyzed. Moreover, it has to be emphasized that this treatment protocol does not induce secondary immune responses but reflects the induction phase of contact hypersensitivity.

RT-PCR

Total RNA was isolated from 10⁷ pooled EC or LNC after adding 1 ml of Trizol reagent (Life Technologies, Grand Island, NY) according to the manufacturer's protocol. The quantity and quality of the extracted RNA were determined by absorbance readings at 280 and 260 nm using a Beckman spectrophotometer (Munich, Germany). To purify extracted RNA from contamination with genomic DNA, a DNase I treatment was performed. Briefly, 2 μ g of RNA was incubated for 20 min at 37°C with 1 μ l of RNasin (40 U; Boehringer Mannheim, Mannheim, Germany), 1 μ l of DNase I (10 U; Boehringer Mannheim), and 4 μ l of 5x reaction buffer in a total volume of 16 μ l. The DNase was inactivated by heating at 90°C for 5 min. Four microliters of oligo(dT)₁₅ primer (0.5 mg/ μ l; Boehringer Mannheim) was added, and the samples were incubated for 5 min at 60°C followed by cooling at 4°C. For reverse transcription, 10 μ l of reaction mixtures containing 1 μ l of RNasin (Boehringer Mannheim), 1.5 μ l of 10 mM dNTP (Boehringer Mannheim), 400 U of Superscript II RT (Life Technologies, Freiburg, Germany), 2 μ l of 5x RT buffer (Life Technologies, Freiburg, Germany), and diethylpyrocarbonate-water were added to a final volume of 30 μ l. RT reactions were conducted at 37°C for 60 min followed by cooling at

4°C.

PCRs were set up using 5 µl of cDNA (equivalent to 200 ng of RNA), 5 µl of 10x amplification buffer, 0.5 µl of 10 mM dNTP, 0.5 µl of each primer (80 mM), 0.5 µl of 5 U/ml Taq polymerase (Boehringer Mannheim), and diethylpyrocarbonate-water to a final volume of 50 µl. This mixture was overlaid with 25 µl of light mineral oil (Sigma). The PCR amplification program was denaturation at 94°C for 15 s, primer annealing at 60°C for 30 s, and extension at 72°C for 30 s. Probes underwent 35 cycles for β -actin and 40 cycles for cytokine analysis. PCRs were performed in a Biometra Trio-bloc thermocycler (Gottingen, Germany).

Competitive template RT-PCR

To semiquantitatively assess cytokine mRNA expression we used a highly sensitive competitive template RT-PCR (21). In this method, a DNA competitor containing the same primer template sequences as the target cDNA competes for primer binding and amplification. To semiquantitate the relative amounts of gene transcripts present in various samples, the individual PCRs were performed by coamplifying the cDNA of interest with an internal PCR control. Differences as low as twofold in cytokine gene expression may be detected by using this competitive template PCR technique (21, 22, 23, 24, 25). The competitor DNA fragment (c.f.) obtained from the plasmid pMCQ contains specific primer sequences for β -actin, IL-1 α , IL-2, IL-4, IFN- γ , and TNF- α (21, 22). Furthermore, a second c.f. (pUV) was constructed that contains the primer sequences specific for IL-1 β (sense primer,

5'-ATGAAAGACGGCACACCCACCCTG-3', position 645–668; antisense primer,

5'-GGCATTAGAAACAGTCCAGCCCAT-3', position 891–914) (26), IL-12 p35 (sense primer,

5'-CAGACAGAGTTCCAGGCCATCAAC-3', position 517–540; antisense primer,

5'-AGGAGCTTAAGCCCACCAGGCCAAG-3', position 851–875) (27), IL-12 p40 (sense primer,

5'-TTGAACTGGCGTTGGAAGCACGGCA-3', position 651–675; antisense primer,

5'-AAGCCAACCAAGCAGAAGACAGCTTT-3', position 1121–1146) (27), and macrophage

inflammatory protein-2 (MIP-2; sense primer, 5'-GAAGTCATAGCCACTCTCAAGGGCG-3', position

242–266; antisense primer, 5'-CAACTCACCTCTCCCCAGAAAC-3', position 695–717) (28), and

granulocyte-macrophage CSF (GM-CSF; sense primer, 5'-GAAGAGGTAGAAGTCGTCTC-3', position

186–205; antisense primer, 5'-GCTGTCTATGAAATCCGCATAGG-3', position 379–401) (29). This

plasmid was cloned into compatible sites of the multicloning region of pBluescript (Stratagene, La Jolla, CA) as described previously (22, 30).

The amplification product of each synthetic competitor differs in size from the original cDNA product (Table I). Using c.f. as an internal standard in RT-PCR allows amplification of both the wild-type original DNA and the c.f. DNA at the same reaction, using gene-specific primers, and separating the products on the basis of size. At similar concentrations of PCR product, comparable band intensities in the ethidium bromide gel electrophoresis will occur. For quantification, cDNA was adjusted to equal concentrations by competitive PCR between β -actin cDNA and c.f. according to a recently described method (22, 23, 25). Each cDNA was amplified in the presence of a fivefold serial dilution of c.f. to estimate equal band intensities of both fragments. Evaluation of competitive RT-PCR experiments was performed on digitalized agarose gels by using image analysis software (RFLPscan software, Sanalytics,

Billerica, MA).

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Table I. Sizes of PCR products of target and control DNA

Primers used for IL-1 α , IL-2, IL-4, IFN- γ , and TNF- α analysis were designed using sequences taken from Murray et al. (31). All primers were provided by Dr. W. Schröder (Pharma Research Center, Bayer, Wuppertal, Germany).

Monoclonal Abs

The following panel of mAbs was used: rat anti-mouse L3T4/CD4 (H129.19, PE, IgG2a) mAb (Boehringer Mannheim) specifically binds to the CD4 Ag. Rat anti-mouse CD25 (AMT-13, FITC, IgG2a) mAb (Boehringer Mannheim) reacts with the α -chain of the mouse IL-2R. Rat anti-mouse CD45R/B220 (RA3-6B2, FITC, IgG2a) mAb (PharMingen, San Diego, CA) recognizes a form of CD45 molecule expressed on B lymphocytes. Rat anti-mouse I-A (M5/114, PE, IgG2b) mAb (Boehringer Mannheim) detects MHC class II Ag. Hamster anti-mouse CD69 (H1.2F3, FITC, IgG) mAb (PharMingen) recognizes the very early activation Ag. Hamster anti-mouse CD80 (16-10A1, FITC, IgG) mAb (PharMingen) specifically binds to the costimulatory molecule B7-1. Hamster anti-mouse CD54 (3E2, FITC, IgG) mAb (PharMingen) reacts with the ICAM-1. For intracellular cytokine staining, rat anti-mouse IFN- γ (XMGI.2, FITC, IgG1) mAb and rat anti-mouse IL-4 (BVD4-1D11, FITC, IgG2b) mAb were used. Polyclonal Abs included PE-conjugated rat IgG2a (R35-95), PE-conjugated rat IgG2b (R35-38), FITC-conjugated rat IgG1 (R3-34), FITC-conjugated rat IgG2a (R35-95), FITC-conjugated rat IgG2b (R35-38), and FITC-conjugated hamster IgG (UC8-4B3, PharMingen).

Flow cytometric analysis of cell surface markers on EC and LNC subpopulations

For double staining of surface Ags, 10⁶ pooled LNC/group ($n = 5$) were incubated with 200 μ l of mAb in PBS, supplemented with 0.02% sodium azide for 30 min at 4°C, washed twice, and analyzed using a FACScan (Becton Dickinson, Mountain View, CA). The following combinations of the above-mentioned mAb were used: anti-CD4 (PE)/anti-CD25 (FITC), anti-CD4 (PE)/anti-CD69 (FITC), anti-I-A (PE)/anti-CD69 (FITC), anti-I-A (PE)/anti-CD54 (FITC), anti-I-A (PE)/anti-CD80 (FITC), and anti-I-A (PE)/anti-CD45R (B220, FITC). PE-conjugated IgG2a, PE-conjugated IgG2b, FITC-conjugated rat IgG2a, and FITC-conjugated hamster IgG served as isotype controls.

Intracellular cytokine staining

Flow cytometry of intracellular cytokine staining was performed as described previously (32, 33). Briefly, for intracellular IFN- γ and IL-4 staining, 10⁶ pooled LNC/group ($n = 5$) were stained with rat anti-mouse CD4 mAb, washed twice, and fixed. Then, cells were permeabilized with saponin buffer (PharMingen), and staining of cytokines was performed by incubating LNC 30 min at 4°C with 100 μ l of saponin buffer

containing FITC-conjugated rat anti-mouse IFN- γ or IL-4 mAb. Afterward, LNC were analyzed using a FACScan (Becton Dickinson).

Statistics

Comparisons were made using the nonparametric Mann-Whitney U test. Differences of $p < 0.05$ were considered significant.

► Results

Characterization of primary contact hypersensitivity responses: increased LNC proliferation and its modulation by topical FK506 treatment

To analyze immunosuppressive mechanisms of action of topical FK506, we first characterized molecular and cellular events during primary contact hypersensitivity responses in murine EC and LNC and secondly analyzed immunomodulatory effects of topical FK506. Kinetic studies showed that optimal sensitization and skin-draining LNC proliferation were induced after contact sensitizer treatment on 3 consecutive days (data not shown). Thus, NMRI mice were topically treated on the dorsal surfaces of both ears with 0.01 to 1% FK506 or vehicle alone on 4 consecutive days. During the last 3 days, ears were additionally painted with the model contact sensitizer, oxazolone (1%). On day 4, local draining lymph nodes of the ears were removed and pooled for each animal, and single cell suspensions were prepared to assess LNC proliferation. Skin painting with 1% oxazolone resulted in significantly increased LNC proliferation, expressed as mean LNC counts ($p < 0.01$). Topical treatment with FK506 (0.01–1%) on 4 consecutive days during the induction phase of contact hypersensitivity dose-dependently suppressed oxazolone-induced LNC proliferation, while FK506 treatment alone did not affect LNC counts compared with those in the vehicle-treated control (Fig. 1□). Furthermore, it could be confirmed that this sensitization protocol reflected the induction phase of contact hypersensitivity and did not include secondary contact hypersensitivity responses. Flow cytometric studies of skin-draining LNC showed initial memory T cell formation (CD4⁺/CD45RA^{low}) on day 5 after the first oxazolone treatment (data not shown). Moreover, adoptive transfer studies reported by van Loveren et al. showed that sensitized T cells could be transferred into recipient mice 4 days after initial sensitization; however, transfer of LNC or spleen cells from donors sensitized 24 or 48 h previously failed to induce challenge-induced contact hypersensitivity responses, indicating that the sensitization protocol used in the present study describes the induction phase of contact hypersensitivity and does not imply secondary responses to sensitized T cells (34, 35).

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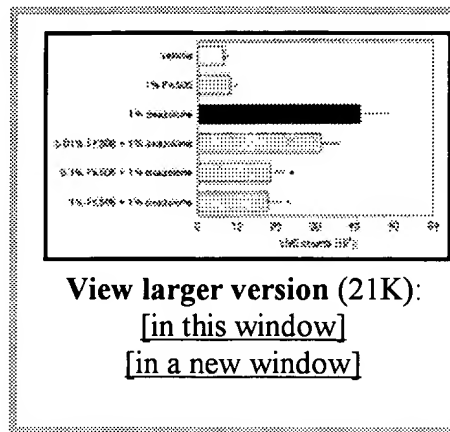


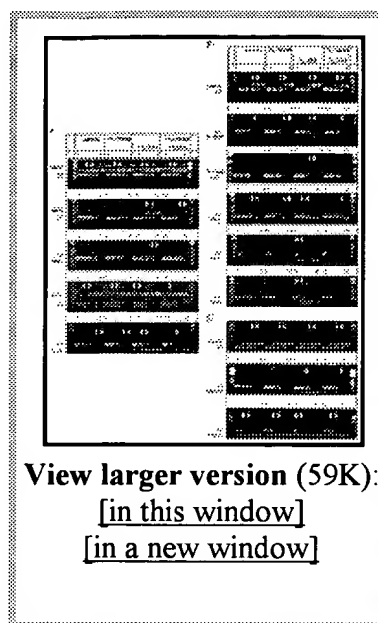
FIGURE 1. Effects of topical FK506 on LNC proliferation during primary contact hypersensitivity responses. Five NMRI mice per group were topically treated with 0.01 to 1% of FK506 on the dorsum of both ears for 4 consecutive days. During the last 3 days, mice received an additional topical treatment with the contact sensitizer oxazolone (1%). On day 5, auricular lymph nodes were removed, and individual LNC counts were measured. Results are expressed as mean LNC counts \pm SD. Statistical significance: * indicates $p < 0.01$ compared with the oxazolone-treated positive control group (by Mann-Whitney U test).

Cytokine mRNA expression in EC and LNC during the induction of contact hypersensitivity

After demonstration of immunosuppressive effects during primary contact hypersensitivity responses, we became interested in the mechanisms of action of topical FK506 in the "local immune system" of the skin. For this purpose, we first analyzed interactions of FK506 with cytokine networks in the epidermis and local draining lymph nodes and secondly investigated corresponding influences on cellular events, such as costimulatory molecule expression on APC, T cell activation and migration into the skin, and associated B cell activation. To investigate the effects of topical FK506 in early and late stages during the induction of contact hypersensitivity, total cellular RNA was extracted from EC and LNC obtained from mice 12 h after the first or 24 h after the third contact sensitizer treatment and analyzed for the expression of IL-1 α , IL-1 β , IL-2, IL-4, IL-12 p35, IL-12 p40, MIP-2, IFN- γ , GM-CSF, and TNF- α transcripts by competitive RT-PCR.

Effects of topical FK506 in an early stage of the induction of contact hypersensitivity

Analysis of epidermal cytokine expression showed that IL-1 α , IL-1 β , GM-CSF, and TNF- α were constitutively expressed in EC and markedly up-regulated 12 h after a single treatment with 1% oxazolone. Additional topical FK506 treatment slightly suppressed the constitutive GM-CSF expression and completely impaired oxazolone-induced up-regulation of both TNF- α and GM-CSF. However, topical FK506 induced only a partial suppression of hapten-induced IL-1 α and IL-1 β expression (Figs. 2 and 3). At this time point no marked differences in cytokine mRNA expression could be detected within skin-draining LNC (data not shown).



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FIGURE 2. Competitive template RT-PCR analysis of cytokine mRNA expression in EC and LNC during primary contact hypersensitivity responses and effects of topical FK506. Fixed concentrations of each target mRNA were coamplified with decreasing concentrations of competitor fragment. Amounts of cDNA calibrated to identical concentrations of β -actin cDNA were coamplified each with fivefold serial dilutions of competitor fragment. The c.f. was diluted to 10^4 , 2000, 400, and 80 molecules/PCR. *Lanes 1 to 5*, Vehicle; *lanes 6 to 10*, FK506; *lanes 11 and 15*, 1% oxazolone; *lanes 16 to 20*, 1% FK506 plus 1% oxazolone. *A*, Analysis of IL-1 α , IL-1 β , IFN- γ , and MIP-2 mRNA expression in EC 3 days after the initial sensitization. *B*, Analysis of IL-12 p35, IL-12 p40, IL-2, IL-4, and IFN- γ in LNC 3 days after the initial sensitization. *C*, Analysis of GM-CSF and TNF- α mRNA expression in EC 12 h after FK506 and oxazolone treatment. Figures show representative data from one of three experiments.

Effects of topical FK506 in a late stage of the induction of contact hypersensitivity: IL-1 α , IL-1 β , MIP-2, and IFN- γ mRNA expression in EC

Although in the epidermis IL-1 α and IL-1 β were constitutively expressed, competitive RT-PCR analysis for MIP-2 and IFN- γ mRNA demonstrated no transcripts in control groups. Primary contact hypersensitivity responses to oxazolone 24 h after the third contact sensitizer treatment were associated with a very strong expression of IL-1 α and IL-1 β mRNA within the epidermis. Furthermore, contact allergen painting induced the expression of MIP-2 and IFN- γ mRNA in EC. Mice topically pretreated with FK506 during the induction of contact hypersensitivity showed a markedly suppressed expression of IL-1 α , IL-1 β , MIP-2, and IFN- γ . Comparison of different cytokines revealed that topical FK506 induced a moderate suppression of IL-1 α and IL-1 β , whereas MIP-2 and IFN- γ mRNA expression was almost abolished (Figs. 2 and 3).

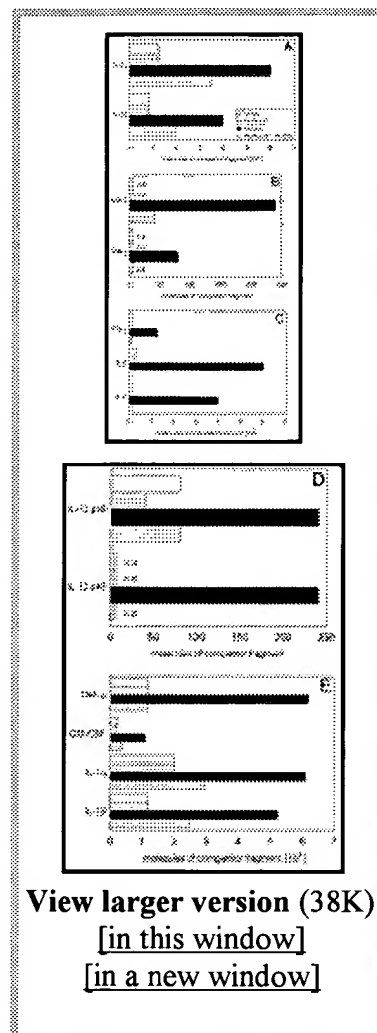


FIGURE 3. The combined results of cytokine mRNA levels. The bars show the amount of competitor fragment molecules that were required to obtain equal band intensities of PCR products from competitor fragment and target cDNA. cDNA of all experimental groups were adjusted to equal concentrations by competitive PCR between β -actin cDNA and the control fragment (see Fig. 2*a-c*). *A* and *B*, Analysis of IL-1 α , IL-1 β , IFN- γ , and MIP-2 mRNA expression in EC 3 days after the initial sensitization. *C* and *D*, Analysis of IL-12 p35, IL-12 p40, IL-2, IL-4, and IFN- γ in LNC 3 days after the initial sensitization. *E*, Analysis of IL-1 α , IL-1 β , GM-CSF, and TNF- α mRNA expression in EC 12 h after FK506 and oxazolone treatment. Figures show representative data from one of three experiments. n.d., not detectable.

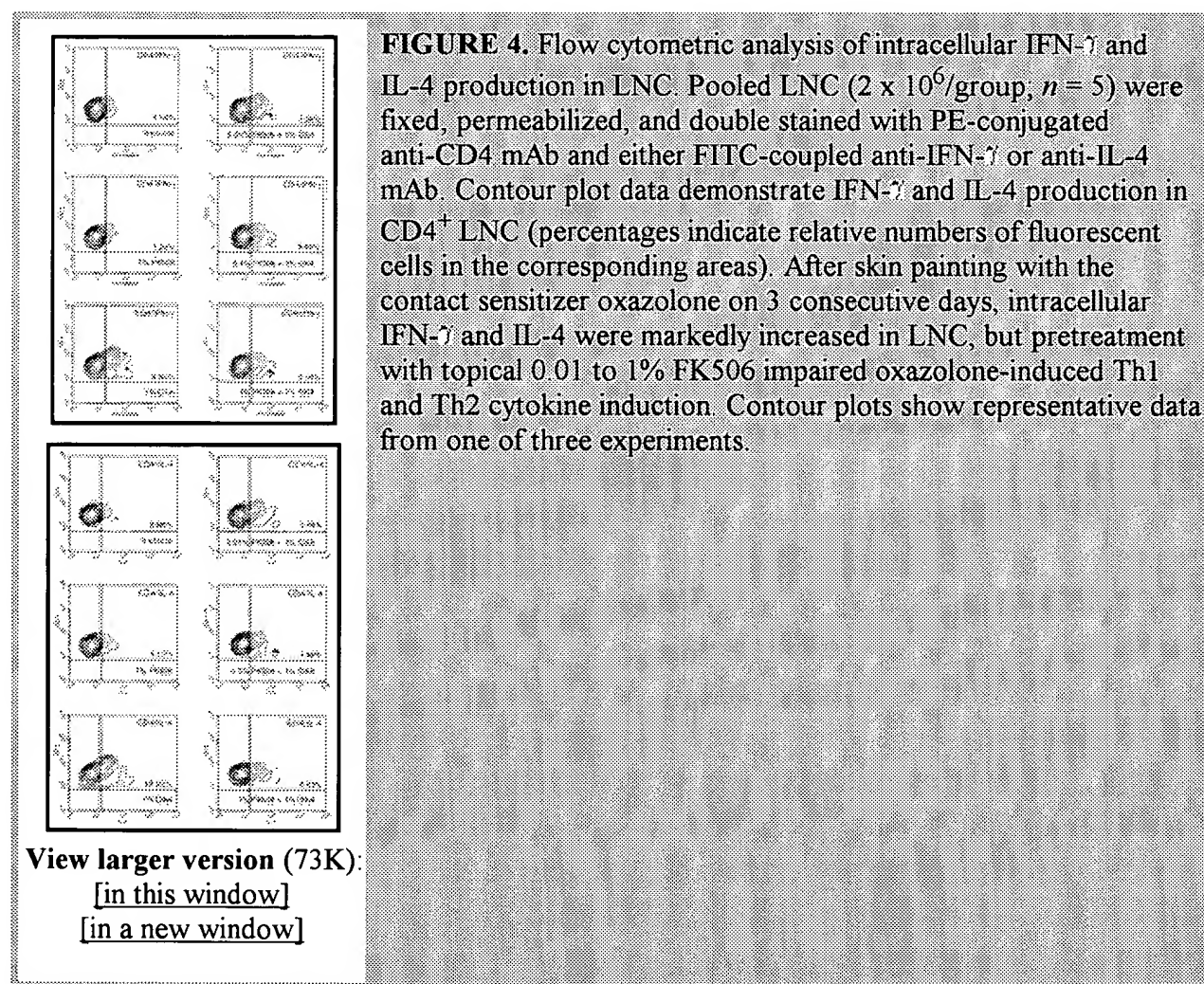
IL-12 p35 and p40 mRNA expression in LNC

Delayed-type hypersensitivity reactions, such as contact hypersensitivity, are considered to be Th1-mediated immune responses (36). Since IL-12 has been shown to be a powerful and probably obligatory factor for the development and proliferation of Th1 cells, we have been interested in whether topical FK506 might modulate IL-12 p35 or p40 mRNA expression (37, 38, 39, 40). Competitive template RT-PCR analysis revealed that IL-12 p35 mRNA was constitutively expressed in LNC and showed a slightly increased expression of IL-12 p35 transcripts in oxazolone-treated groups compared with that in vehicle-treated controls. Topical FK506 treatment suppressed oxazolone-induced up-regulation of IL-12 p35 mRNA expression down to the level in vehicle-treated controls. Interestingly, constitutive IL-12 p35 mRNA expression in LNC was marginally suppressed after FK506 treatment alone. In contrast, no constitutive expression of IL-12 p40 mRNA could be detected, but significantly increased expression was induced in LNC by oxazolone. However, pretreatment with topical FK506 completely abolished inducible IL-12 p40 mRNA expression during primary contact hypersensitivity responses (Figs. 2*d* and 3*d*).

Th1 and Th2 cytokine expression in LNC

LNC proliferation during the induction phase of contact hypersensitivity was associated with an increased expression of both Th1-type cytokines, such as IL-2 and IFN- γ , and the Th2-type cytokine IL-4 compared with cytokine mRNA expression in control mice. The dose-dependent suppression of oxazolone-induced LNC proliferation in mice topically treated with FK506 was accompanied by a marked inhibition of IL-2, IFN- γ , and IL-4 mRNA expression (Fig. 2*e*).

To confirm our results obtained at the mRNA level, we performed flow cytometric analysis of intracellular cytokine production in LNC. Induction of both Th1-type cytokine IFN- γ and Th2-type cytokine IL-4 in CD4⁺ LNC could be detected after skin painting with oxazolone. Pretreatment with topical FK506 resulted in a dose-dependent suppression of both cytokines. For IL-4 induction, a plateau of suppression was reached at 0.1% FK506 (Fig. 4*e*).



Characterization of primary contact hypersensitivity responses: cellular events within the epidermis and local draining lymph nodes

To demonstrate the relevance of the molecular findings, we determined the corresponding cellular effects of the cytokines analyzed. Biologic effects of IL-1 α , IL-1 β , TNF- α , and GM-CSF expression in EC were

assessed by analyzing epidermal APC activation and maturation. To determine the cellular effects of MIP-2, we analyzed contact allergen-induced infiltration of CD4⁺ T cells into the epidermis. Furthermore, skin-infiltrating CD4⁺ T cells have been shown to be the main source of epidermis-derived IFN- γ (41). Moreover, IL-2-driven T cell activation was assessed by measuring IL-2R α -chain expression on CD4⁺ LNC, and associated B cell activation was determined as a marker for IL-4 induction.

Although on day 3 after initial sensitization with oxazolone significant changes in cell surface marker expression could be detected in both EC and local draining LNC, 12 h after the first contact sensitizer treatment no differences were seen.

MHC class II molecule (I-A) expression in murine EC

Skin painting with the contact sensitizer oxazolone on 3 consecutive days induced markedly increased expression of MHC class II molecules (I-A) on the surface of murine EC compared with that in vehicle-treated controls. Pretreatment with topical FK506 during oxazolone-induced contact hypersensitivity resulted in a partial suppression of I-A⁺ EC subpopulations, whereas FK506 treatment alone revealed no significant difference from vehicle-painted control animals (Table II \square).

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Table II. Flow cytometric analysis of cell surface marker expression in EC and LNC during primary contact hypersensitivity responses and influences of topical FK506 treatment^a

Costimulatory molecule expression on I-A⁺ EC and LNC

EC and LNC were double stained with either anti-CD54 (ICAM-1) or anti-CD80 (B7-1) and anti-I-A mAb. After skin painting with oxazolone, a markedly increased expression of both CD54 and CD80 could be detected on the surface of I-A⁺ EC. Vehicle-treated mice expressed almost no costimulatory molecules on I-A⁺ EC, but during primary contact hypersensitivity responses, CD54 and CD80 were significantly up-regulated, whereas mice pretreated with 1% FK506 showed markedly impaired costimulatory molecule expression. Similarly, costimulatory molecules were up-regulated on I-A⁺ LNC after oxazolone treatment and markedly suppressed after topical FK506 pretreatment (Table II \square).

T cell migration and T cell activation

T cells play an essential role in inflammatory skin diseases, where they are implicated in the pathogenesis of atopic eczema and psoriasis (42, 43, 44). Thus, we have been interested in modulatory effects of topical FK506 on T cell activation and epidermal T cell recruitment. T cell migration was measured during the induction of contact hypersensitivity as a function of the appearance of CD4⁺ cells within the epidermis. Topical treatment with FK506 markedly suppressed oxazolone-induced CD4 expression in EC suspensions, indicating impaired epidermal T cell recruitment. In good correspondence to profoundly decreased IL-2 mRNA expression, oxazolone-induced up-regulation of the IL-2R α -chain (CD25) on CD4⁺ LNC was dramatically suppressed below control values after FK506 treatment (Table II \square).

Associated B cell activation

Flow cytometric analysis revealed a markedly increased expression of MHC class II molecules (I-A) on B220⁺ LNC during oxazolone-induced contact hypersensitivity responses. In parallel, the very early activation Ag, CD69, was dramatically overexpressed on I-A⁺ LNC after contact allergen painting. Interestingly, pretreatment with topical FK506 during primary contact hypersensitivity responses resulted in a clear suppression of both I-A⁺/B220⁺ and I-A⁺/CD69⁺ LNC subsets (Table II \square).

Discussion

The principle findings of this study concerning the effects of topical FK506 treatment during early and late stages of primary contact hypersensitivity responses in mice are as follows: 1) suppression of primary (IL-1 α , IL-1 β , and TNF- α) and secondary (GM-CSF and MIP-2) epidermal cytokine mRNA expression; 2) perturbed IL-12 p35 and p40 mRNA expression in local draining LNC accompanied by impaired induction of both Th1 (IL-2 and IFN- γ) and Th2 (IL-4) cytokines; 3) suppression of costimulatory molecule (CD80 and CD54) expression on I-A⁺ EC and LNC indicating impaired APC activation and maturation; 4) profound suppression of T cell activation and T cell migration; and 5) suppression of associated B cell activation.

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Enk and Katz have characterized early molecular events during the induction phase of contact hypersensitivity in EC and found that several epidermis-derived cytokines, such as IL-1 α , IL-1 β , MIP-2, TNF- α , and T cell-derived IFN- γ were up-regulated within minutes or hours after contact allergen treatment (41). Additional experiments demonstrated that IL-1 β subserved an essential function for the induction of contact hypersensitivity reactions (45). Furthermore, transgenic mice that overexpress the primary cytokines IL-1 α and TNF- α in basal epidermis develop inflammation and skin disease (46). These findings indicate a crucial role for IL-1 and TNF- α in the induction of primary skin immune responses. Our results confirm the up-regulation of primary and secondary epidermis-derived cytokines during the induction of contact hypersensitivity and demonstrate markedly suppressed IL-1 α , IL-1 β , GM-CSF, TNF- α , MIP-2, and IFN- γ expression due to concomitant topical FK506 treatment. Comparison of different epidermis-derived cytokines revealed that topical FK506 caused a complete suppression of hapten-induced TNF- α and GM-CSF up-regulation, and a partial suppression of IL-1 α and IL-1 β expression. Moreover, mRNA expression of the mouse operational homologue to human IL-8, MIP-2, and mRNA expression of T cell derived IFN- γ were almost abolished in EC after concomitant topical FK506 treatment. Studies performed 12 h after the first oxazolone treatment confirmed the suppression of primary and secondary epidermal cytokine expression by topical FK506 and suggest that these effects are not a secondary phenomenon due to the inhibition of T cell cytokine production.

Furthermore, our results obtained in vivo correlate well with recently published in vitro studies. Keisho and coworkers demonstrated that FK506 partially suppressed IL-1 α production in macrophage-like U937 cells, human monocytes, and alveolar macrophages in a dose-dependent manner, while Reismann et al.

showed that FK506, cyclosporin A, and rapamycin only partially suppressed IL-1 β secretion in THPI cells (47, 48). Moreover, our group previously showed that FK506 is a potent suppressor of IL-8 and IL-8R expression in cultured human keratinocytes (8, 11).

To demonstrate the relevance of the molecular findings, we determined the corresponding biologic effects of IL-1 α , IL-1 β , TNF- α , and GM-CSF mRNA expression in EC by analyzing activation and maturation of APC. During contact hypersensitivity, IL-1 α , IL-1 β , TNF- α , and GM-CSF regulate activation and maturation of skin-related APC, resulting in increased MHC class II molecule and costimulatory molecule expression (45, 49, 50). Flow cytometric analysis of I-A expression on murine EC during primary contact hypersensitivity responses showed that up-regulation of both I-A and costimulatory molecule (CD54 and CD80) expression was moderately suppressed by topical FK506 treatment. Thus, molecular findings regarding IL-1 α , IL-1 β , TNF- α , and GM-CSF mRNA expression were in accordance with I-A expression on EC.

With respect to the cellular effects of the keratinocyte-derived chemokine, MIP-2, contact allergen-induced epidermal T cell recruitment has been analyzed by flow cytometry (41, 51). Furthermore, Enk and Katz previously showed that skin-infiltrating T cells are the main source of epidermis-derived IFN- γ (41). Here, we could demonstrate the infiltration of CD4⁺ T cells into the epidermis and parallel the expression of epidermis-derived IFN- γ during the induction phase of contact hypersensitivity. Consistent with molecular findings, flow cytometric analysis showed that epidermal T cell recruitment was clearly suppressed after additional topical FK506 treatment.

Recently, it has been demonstrated that besides IL-1, the 70-kDa heterodimeric cytokine, IL-12, which is composed of a constitutively expressed p35 subunit and an inducible p40 subunit, also plays a critical role in the initiation of primary immune responses (37, 38, 39, 40). Further evidence for the important role of IL-12 during primary immune responses was provided by the work of Müller et al. and Schwarz et al. who recently demonstrated that i.p. injection of IL-12 breaks UV-induced tolerance in contact allergen-sensitized mice and correspondingly showed that neutralization of IL-12 prevented dinitrofluorobenzene-induced contact hypersensitivity and induced hapten-specific tolerance (52, 53, 54). Therefore, we have been interested whether topical FK506 might influence IL-12 p35 or p40 expression during primary contact hypersensitivity responses. In contrast to the p35 subunit, which was constitutively expressed in LNC, expression of the p40 subunit could only be detected after contact allergen exposure. Interestingly, topical FK506 treatment during the induction phase of contact hypersensitivity significantly suppressed up-regulation of IL-12 p35 and abolished IL-12 p40 mRNA expression in local draining LNC. After demonstrating IL-12 up-regulation and modulation, we investigated the cytokine pattern induced in LNC during primary contact hypersensitivity responses. Both competitive RT-PCR analysis and intracellular cytokine staining revealed that LNC exhibit a Th0 phenotype, with both Th1 (IL-2 and IFN- γ) and Th2 (IL-4) cytokine induction during primary contact hypersensitivity responses. This finding is consistent with studies of Fehr et al. and Hope et al., who demonstrated production of both Th1 and Th2 cytokines during hapten-induced contact hypersensitivity (55, 56). Pretreatment with topical FK506 substantially impaired contact allergen-induced Th1 (IL-2 and IFN- γ) and Th2 (IL-4) cytokine induction in LNC at both the mRNA and the protein level. Impaired Th1 and Th2 cytokine induction may be linked to direct inhibition of NF- κ B by topical FK506 (57, 58).

Moreover, suppressed IL-12 mRNA expression will affect Th1 cytokine expression during primary contact hypersensitivity responses (37).

Next to soluble factors, such as cytokines, costimulatory molecules play an essential role in primary immune responses. Recently, it has been demonstrated that the signal derived from the engagement of TCR alone is not only insufficient for optimal T cell activation, but also leads to Ag-specific unresponsiveness, or anergy (59, 60, 61). A second costimulatory signal determines in an Ag-independent manner whether the stimulated T cells proliferate or become anergic. Several recent studies have provided evidence that B7 molecules (CD80 and CD86) or ICAM-1 (CD54) can transduce such costimulatory signals through interaction with CD28 or LFA-1 (62, 63, 64, 65). The results of the present study show that contact allergen exposure resulted in significantly increased I-A⁺/CD80⁺ and I-A⁺/CD54⁺ EC and local draining LNC subsets, indicating APC activation and maturation during primary contact hypersensitivity responses. Both contact allergen-induced CD54 and CD80 up-regulation on I-A⁺ EC and LNC were significantly decreased after topical FK506 pretreatment. Moreover, recent in vitro findings with human epidermal Langerhans cells reported by Panhans et al. confirmed our in vivo results showing that FK506 suppresses costimulatory molecule (e.g., CD80) expression. The stimulatory capacity of FK506-treated Langerhans cells was dose-dependently decreased, confirming the functional relevance of these in vitro phenomena (66). In aggregate, these findings suggest that FK506 exerts direct effects on non-T cells and may induce Ag-specific tolerance or anergy by suppressing costimulatory molecule expression on the surface of APC.

During the afferent phase of contact hypersensitivity, sufficient presentation of Ag to T cells in local draining lymph nodes results in Ag-specific T cell proliferation and differentiation that is driven by IL-2 (67). Since the presence of IL-2 is one of the major inducers of IL-2R expression and clonal expansion of T cells, profound suppression of IL-2 mRNA expression due to topical FK506 treatment correlates well with the dramatically impaired IL-2R expression on CD4⁺ LNC and decreased LNC proliferation.

Although delayed-type hypersensitivity responses have been demonstrated to be T cell dependent, characterization of a Th0 cytokine pattern in local draining LNC prompted us to analyze lymph node B cell subsets during the induction phase of contact hypersensitivity. With respect to cytokine-mediated B cell activation, IL-4 plays a major role. Incubation with this "activation factor" showed a striking increase in the density of MHC class II molecules on B cells (68). Flow cytometric analysis of LNC revealed that contact allergen painting was accompanied by an expansion of the I-A⁺/B220⁺ LNC subpopulation. Furthermore, a dramatically increased expression of the very early activation Ag CD69 could be demonstrated on I-A⁺ LNC. These results provide evidence that lymph node enlargement and LNC proliferation during primary contact hypersensitivity responses is linked to both T cell and associated B cell activation and proliferation. Our results suggest that associated B cell activation is linked to IL-4 up-regulation because dramatically suppressed IL-4 induction by topical FK506 was correlated with decreased I-A⁺/B220⁺ and I-A⁺/CD69⁺ LNC subpopulations.

In summary, this study extends previous in vitro findings, in which FK506 suppressed T cell-derived cytokine expression via inhibition of the transcription factor NF-AT (57, 58). We demonstrate for the first time immunologic changes after topical application of the drug, which is of particular interest for the

clinical situation.

Indeed, topical FK506 might open a new era in the treatment of inflammatory skin diseases. In this respect, topical FK506 may be used as a tool to suppress both Th1 and Th2 cytokine production. This idea may provide a clue to explain the success of topical FK506 therapy to treat atopic eczema ([5](#), [6](#), [7](#)). It might be postulated that topical FK506 can interrupt the self-sustaining cycle of hyperactivation in atopic dermatitis by regulating Th1/Th2 imbalance, by modulating T cell migration via decreased chemokine expression and by impairing costimulatory molecule (CD54 and CD80) expression on APC ([42](#), [43](#), [66](#), [69](#), [70](#)).

Footnotes

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² Abbreviations used in this paper: EC, epidermal cell; LNC, lymph node cell; c.f., competitor DNA fragment; MIP-2, macrophage inflammatory protein-2; GM-CSF, granulocyte-macrophage colony-stimulating factor; PE, phycoerythrin; ^{low}, low level. [↑](#)

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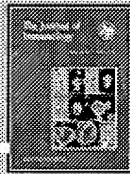
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